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Effects of Long- and Short-Term Passage of Insect Cells in Different Culture Media on Baculovirus Replication

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Two insect cell lines that had been maintained in both serum-free (SFM) and serum-containing (SCM) media for over 5 years were each tested for their ability to replicate baculovirus. The gypsy moth cell line, IPLB-LdEIta (Ld), produced similar (not statistically different) amounts of gypsy moth nucleopolyhedrovirus (LdMNPV) occlusion bodies (OBs) in the two media (serum-free Ex-Cell 400 and TC-100 with 9% (v/v) fetal bovine serum, SCM_1) but produced more of the *Autographa californica* nucleopolyhedrovirus (AcMNPV) OBs in SFM than in SCM_1. When Ld cells normally grown in SCM_1 were switched to SFM, production of OBs from both viruses improved and, after three passages, reached higher levels of AcMNPV production than in cells normally maintained in that medium. Alternatively, cells switched from SFM to SCM_1 initially produced as much (in the case of LdMNPV) or higher (in the case of AcMNPV) levels of virus OBs than cells normally maintained in SCM_1 but productivity dropped off over subsequent passages such that after five passages in SCM_1, cells produced substantially fewer OBs of both viruses. A fall armyworm cell line (IPLB-SF21AE; Sf) showed slightly different effects from long- and short-term passage in SFM (Ex-Cell 400) or SCM_2 (TMN-FH). Cells maintained in SFM produced about 20 times more AcMNPV OBs than cells maintained long-term in SCM. Sf cells switched from SFM to SCM maintained the level of production of that seen in SFM at the first passage, but quickly dropped off OB production levels to that normally seen in SCM. Alternatively, SCM-maintained Sf cells produced higher levels at the first passage in SFM and, within five passages in SFM, reached levels found in cells maintained for long term in this medium. Under the conditions in which these two cell lines were infected, the highest levels of AcMNPV OB production in Ld cells were about five times that of Sf cells. In a separate series of experiments, cells normally grown in SFM were passaged over five times in Ex-Cell 400 to which serum was added; both cell lines produced as much virus as that in SFM. These results suggest that it is not the serum *per se* but rather some other components which differ between the SFM and the SCM formulations that are responsible for the varied virus production obtained in these studies. The results of these studies suggest that a maintenance and virus production protocol can be developed with Ld cells which could improve overall efficiency of virus production. These studies also suggest that long-term maintenance of cells in SFM was not detrimental to their ability to produce baculoviruses.

***Key Words: Lymantria dispar; Spodoptera frugiperda;* nucleopolyhedrovirus; AcMNPV; LdMNPV; cell cultures; media effects; virus productivity.**

INTRODUCTION

The culture medium used for insect cells has been known to affect baculovirus production since the earliest days of this research field (Stockdale and Gardiner, 1975; Goodwin and Adams, 1980). Much of the early effort to improve media was fueled by plans to produce insect viruses in cell cultures as viral pesticides. The field received much more attention, however, with the advent of the baculovirus expression vector system (Smith *et al.*, 1983) and its subsequent common use for producing proteins of pharmaceutical importance (over 500 proteins have been produced; Patterson *et al.*, 1995). Since the early work was focused on decreasing the cost of virus production, researchers often used inexpensive, undefined ingredients, whereas the later work with expression vectors demanded defined ingredients to simplify purification of the recombinant protein and to satisfy regulatory agencies as to the purity of the final product. Success has been achieved to some extent in both areas. Mitsuhashi (1989) and Vaughn and Fan (1997) have each developed simple, inexpensive media that support baculovirus replication, whereas Wilkie *et al.* (1980) and Mairorella *et al.* (1988) created largely defined formula. A recent review by Schlaeger (1996) presents more details on the history of insect cell culture media. In recent years, several commercial media producers have developed and

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marketed serum-free, low-protein media for insect cell use in expression vectors.

I tested the first of these commercial serum-free media (SFM), Ex-Cell 400 (JRH Biosciences, Lenexa, KS), for it's ability to grow a number of insect cell lines from different species. As would be expected, it grew *Spodoptera frugiperda* cells (the cells used in its development) very well, but also supported growth of cell lines from *Lymantria dispar* (four lines), *Trichoplusia ni, Trichogramma exiguum, Trichogramma confusum,* and *Diabrotica undecimpunctata.* At the same time, other lines from *T. ni* and other Lepidoptera grew poorly or not at all on this medium (unpublished data).

The influence on virus production of long-term culture of cells under different conditions has been largely unexplored, but it seemed reasonable to expect maintaining cells on different media (especially media with and without serum) could affect virus productivity, either positively or adversely. Because of this potential, I have maintained two lines, IPLB-Sf21AE from *S. frugiperda* (Vaughn *et al.,* 1978) and IPLB-LdEIta from *L. dispar* (Lynn *et al.*, 1988), on both a serum-containing medium (SCM) and SFM continuously for over 5 years. In a recent report (Lynn, 1999), I showed that there was little difference in the number of *Autographa californica* nucleopolyhedrovirus (AcMNPV) viral occlusion bodies (OBs) produced by cells from one of these lines (LdEIta) maintained for a long period of time in SCM compared with that of the SFM cells. However, simply switching cells to the alternate medium actually improved virus production in both cell strains (that is, cells maintained in SFM produced more virus when infected in SCM and cells maintained in SCM also produced more when infected in SFM). Productivity was also improved when the cells were switched to the alternate medium and infected with a different virus (the *L. dispar* nucleopolyhedrovirus, LdMNPV).

In the current study, I extend these results to a second cell line (SF21AE) and test whether improved virus production is maintained for subsequent subcultures in the alternate medium.

MATERIALS AND METHODS

Cell lines and media. IPLB-LdEIta cells were maintained continuously on modified TC-100 for over 270 weekly subcultures (Ld-s) or Ex-Cell 400 for over 500 weekly subcultures (Ld-x) as described previously (Lynn, 1999). IPLB-SF21AE cells were maintained continuously on modified TNM-FH (Hink and Strauss, 1976) for over 380 weekly subcultures at approximately a 1:20 split ratio (Sf-s) or Ex-Cell 400 for over 430 weekly subcultures at approximately a 1:40 split ratio (Sf-x). In the experiments examining the effects of short-term passage of the cells, each cell strain was maintained for up to 10 weeks on the alternate medium. Thus, Ld-s and Sf-s were grown in Ex-Cell 400 and Ld-x and Sf-x were grown on TC-100 or TNM-FH, respectively. Ld-x and Sf-x were also passaged over five times in Ex-Cell 400 to which 9% (v/v) fetal bovine serum (FBS) was added. The split ratios were adjusted during these subcultures so that cells reached confluence at the end of 7 days. The identity of each of these cell strains (as well as all other cell lines maintained in my laboratory) was confirmed annually by isozyme analysis (Lynn, 1996).

Virus studies. Virus inocula of clones of AcMNPV (ACMNPV-pxh) and LdMNPV (LdMNPV-a624) were as described previously (Lynn, 1999). Cells of each strain were counted *in situ* using a Nikon TMS inverted microscope fitted with a calibrated reticulated eyepiece. Cells were suspended by flushing with medium from a transfer pipet, centrifuged (50*g*, 5 min), and then resuspended at 5×10^4 (Ld-s and Ld-x) or 1×10^5 (Sf-s and Sf-x) cells per milliliter of the appropriate medium. One milliliter of cells was added to each well of a 24-well plate (Costar, Cambridge, MA). Immediately after the plates were inoculated with cells, each well was inoculated with virus at a multiplicity of infection of 5 $TCID_{50}$/cell (virus inocula titers were determined in IPLB-LdFBc1 (LdMNPV-a624) and IPLB-TN-R^2 (AcMNPV-pxh) cells, respectively, as described previously; Lynn, 1999). Four replicate wells were set up for each cell/virus/medium combination. *L. dispar* cells were inoculated with both viruses but the *S. frugiperda* cells were infected only with AcMNPV-pxh since they are not susceptible to LdMNPV. After infection, plates were stored at 22°C in the dark. A week after inoculation, 0.8 ml of the medium from each well was carefully removed to avoid disturbing the cells. One milliliter of sterile demineralized water was added followed by 50 μl of a 10% (w/v) sodium laurel sulfate (Sigma, St. Louis, MO) solution. The cells were suspended and lysed by multiple pipettings through a Pasteur pipet (Corning Glass, Corning, NY). The plates were left at room temperature overnight to allow the OBs to sediment and OBs were counted with the inverted microscope as described above. The resulting counts were used to estimate the number of OBs produced in each well. The data was analyzed by analysis of variance and Tukey's pairwise multiple comparison test using Sigmastat (v 2.03) on the log of the OB/ml estimates.

RESULTS

Cell growth rates differ for each of these cell lines in the two media that were used in the tests as shown in Fig. 1. The IPLB-LdEIta cells grew best in the SFM (Ex-Cell 400), having approximately a 7-fold increase over a 7-day period at 25°C, whereas cells in SCM_1 (TC-100) only doubled in 7 days. Alternatively, IPLB-Sf21AE cells grew best in SCM_2 (TNM-FH), showing a 40-fold increase over a week relative to an approxi-

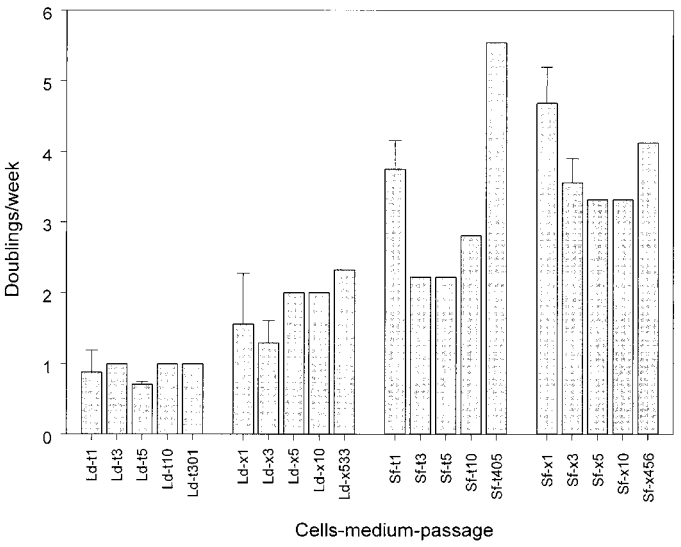

FIG. 1. Cell culture doublings in 1 week on Ex-Cell 400 (SFM) or TC-100 (SCM_1) or TNM-FH (SCM_2) after various lengths of times that each cell line was maintained on each medium. X axis labels represent the cell line (Ld, LdEita cells; Sf, Sf21AE cells), medium (s, serum-containing, modified TC-100 medium for Ld and TNM-FH for Sf; x, serum-free Ex-Cell 400), and number of passages in that particular medium since transfer from the alternate medium. The bars are the means and error bars are standard deviations based on the split ratios. The number of counts for each bar varied depending on how many times the cells were passaged.

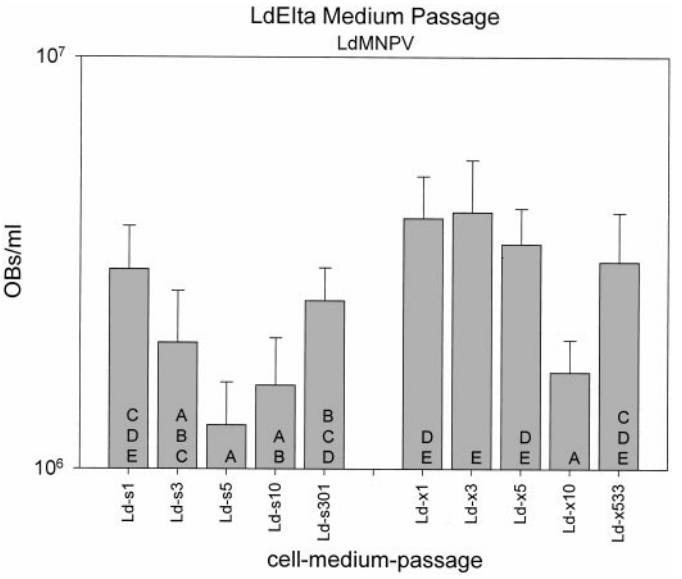

FIG. 2. Productivity of gypsy moth virus (LdMNPV-a624) in gypsy moth cells (IPLB-LdEIta) in two media over a series of 10 passages or at very high passage. X axis labels are as described for Fig. 1. Each bar represents the mean number of occlusion bodies produced per milliliter of media in four replicate cultures. The error bars are one standard deviation and bars labeled with the same letter are not significantly different ($P = 0.05$) as determined by Tukey's mean comparison test.

mately 20-fold increase for the same line maintained in SFM. These numbers reveal that the SF21AE cells have a substantially faster growth rate regardless of the medium. The SF21AE cells also reach a higher final cell density (2–3 $\times$ 10^6 cells/ml in routine culture compared with 0.8–1.2 $\times$ 10^6 for the LdEIta cells), although the LdEIta cells are much larger cells with diameters of 40–82 μm compared to 15–20 μm for SF21AE; so, these maximum densities represent confluent cell monolayers in each case. Figure 1 also reveals that the growth rates upon switching the cells from one medium to the other slowly revert to being equivalent to how cells grow in the new medium at high passages. That is, SF21AE cells switched from SCM_2 to SFM initially grow faster than cells normally maintained in SFM, but over five passages become similar to the high-passage SFM, whereas SF21AE cells switched from SFM to SCM_2 grew slower initially, but within five passages were growing as well as cells maintained in SCM_2. Similar growth rate changes were observed with LdEIta cells.

The productivity of virus in the respective cells and media at various passage levels as represented by OB production is shown in Figs. 2–4. Figures 2 and 3 show LdMNPV-a624 and AcMNPV-pxh production, respectively, in LdEIta cells and Fig. 4 shows AcMNPV-pxh production in Sf21AE cells. The results with LdEIta cells are similar to previous results (Lynn, 1999) using this cell line in which cells maintained for a long term in SCM_1, represented in Figs. 2 and 3 by the bars labeled Ld-s301, produced more virus when switched to SFM (Ex-Cell 400; represented in Figs. 2 and 3 by Ld-x1). The cells normally maintained in SFM (x533) did not produce more in these experiments when switched to SCM_1 (Ld-s1). Statistically, there was no difference between OB production in high-passage

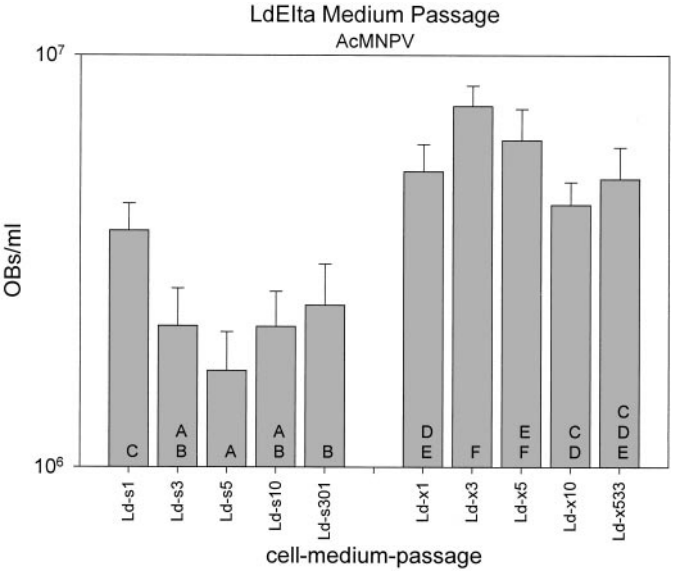

FIG. 3. Productivity of *A. californica* virus (AcMNPV-pxh) in gypsy moth cells (IPLB-LdEita). The results are presented as described in Fig. 2.

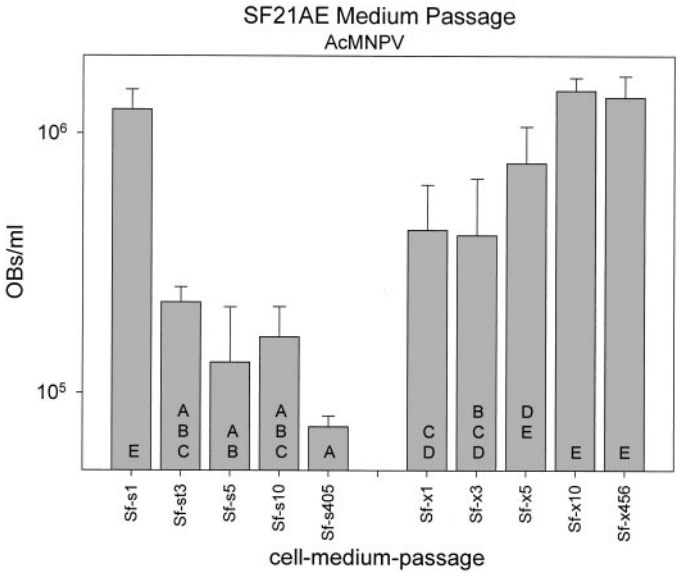

FIG. 4. Productivity of AcMNPV-pxh virus in fall armyworm cells (IPLB-Sf21AE). The results are presented as described in Fig. 2.

SFM (Ld-x533) and that in the first passage in SCM_1 (Ld-s1).

The stimulatory effects on virus production of switching from SCM_1 to SFM were maintained for several passages in the new medium in LdEIta cells (as represented by bars labeled Ld-x3 and Ld-x5 in Figs. 2 and 3). This was true with both viruses, with AcMNPV OB production actually increasing at passage 3 before declining to the level achieved in high-passage SFM. Alternatively, by passage 10 in SFM, LdMNPV virus production declined to the lowest levels in this medium.

Another observation that can be made from Figs. 2 and 3 is that SCM_1 cells produced lower quantities of virus at nearly every passage level. In cells infected with either virus, production levels declined to the lowest level after 5 passages in SCM_1 (Ld-s5 in both figures) but then appeared to recover by the 10th passage.

Although the effects of changing from SCM to SFM and vice versa in IPLB-SF21AE cells appear similar to those observed with LdEIta at the first passage in the new medium (that is, cells switched from SCM_2 (TNM-FH; bar marked Sf-s405 in Fig. 4) to SFM (Ex-Cell 400, Sf-x1) produced more virus and cells switched from SFM (Sf-x456) to SCM_2 (Sf-s1) did not change significantly), the effects were different over subsequent passages. The production of AcMNPV OBs steadily improved in subsequent passages in SFM such that there was no significant difference with long-term passage in SFM by passage five (Sf-x5 compared with Sf-x456), whereas production in SCM_2 quickly declined so that there was no significant difference at passage three (Sf-s3) from the long-term passage in SCM_2 (Sf-s405).

In a separate experiment, each cell line was also passaged over five times in Ex-Cell 400 to which 9% FBS was added. In these tests, both cell lines produced amounts of virus equivalent to that of SFM and greater amounts than that of long-term growth in the respective SCMs (data not shown).

DISCUSSION

The formulation of culture media can play a significant role in the production of virus or recombinant proteins in insect cells. I have previously shown that, for one gypsy moth cell line (Lynn, 1999), virus production increased not only when cells were switched from medium containing serum to SFM, but also when cells were switched from SFM to SCM. This suggested that it was not just a matter of simple nutrition, but that some stimulatory effect was achieved by the change in media. These results were extended in this current study by extending the length of time that the cells were kept in the alternate medium to 10 passages. These tests suggest that maintaining the gypsy moth cells for even a few passages resulted in a return to production levels obtained from very high passages. In the case of cells switched from SFM to SCM, productivity of OBs actually declined at passage 5 to levels below that obtained from cells normally maintained in that medium. The productivity subsequently improved so that by the 10th passage, there was no significant difference from that of cells normally kept in that medium. These results were the same for both viruses tested (AcMNPV and LdMNPV).

Tests with IPLB-SF21AE cells in this study also suggest that switching cells which have been maintained for a long term in a medium with serum to SFM resulted in improved OB production but not to as great a level as that obtained from cells already maintained in the SFM formulation. Also, unlike the earlier results with gypsy moth cells, SF21AE cells normally maintained in SFM did not produce higher levels of virus when switched to SCM_2. However, within a few passages, productivity was not different between low-passage and high-passage cells in either medium. Simple nutritional effects can easily explain these results if the SFM is more nutritionally complete than the SCM_2. The experiments in which serum was added to the SFM formulation also supports this since both cell lines produced as much virus over five passages as that in long-term SFM and significantly more than cells maintained in the respective SCM.

The other information that can be gleaned from these studies is that, under the conditions used in these tests, the LdEIta cells produced greater levels of AcMNPV OBs. Figure 5 is a composite of data from Figs. 3 and 4 showing that the maximum OB production in LdEIta cells was more than five times as much as the maximum obtained from SF21AE. Note that these results are based on infecting each cell line at a relatively low cell density and immediately after cells are placed in fresh culture medium. In some other tests, SF21AE cells inoculated 24 h after the plates

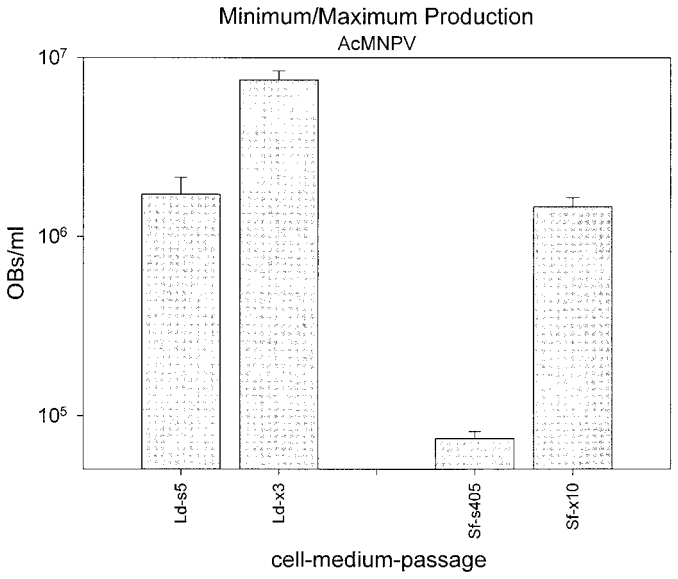

FIG. 5. Data from Figs. 3 and 4 representing the highest and lowest amount of AcMNPV-pxh virus produced in the two cell lines present here for direct comparison between the two cell systems. The results are presented as described in Fig. 2.

were initiated produced fewer OBs in most media/passage levels than the same cells that were inoculated at time 0 (data not shown). Cells should not have depleted any nutrients at this point; so, this difference in productivity is probably due to some stimulatory effect of fresh medium or the subculture procedure on the cells. The LdEIta cells did not show a similar difference between cells inoculated at 24 h compared with time 0.

The greater production by LdEIta reported here is similar to results obtained by other researchers with recombinant AcMNPV in which β-galactosidase, rotavirus VP4 protein, and/or human plasminogen was produced at a higher level in these cells than in SF21AE or a clone (Hink *et al.*, 1991; Betenbaugh *et al.*, 1991). Since AcMNPV OB production was maximal in LdEIta after three passages in SFM, it would be possible in a production scheme to maintain the stock of these cells in SCM but do the final scale-up and production in SFM. This would maximize production while maintaining the advantage of the SFM for purification and recovery of the final product. A major drawback of the LdEIta cells is their slower growth. In my hands in stationary cultures, they currently double on a 2- to 7-day cycle, depending upon the medium, whereas SF21AE cells double in less than a day (Fig. 1). Thus, if time was a factor, more product can be produced in SF21AE in the same time frame but with a greater expenditure in medium.

This study also reinforces my earlier results (Lynn, 1999), which suggest that long-term maintenance of insect cells in SFM is not detrimental to their ability to produce nucleopolyhedroviruses.

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